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14. ABSTRACT

Early studies from our laboratory found that beta2-chimaerin has a Rac-GAP (GTPase-Activating Protein) domain and negatively regulates the activity of Rac, an important small GTPase for the control of cytoskeleton reorganization, cell cycle progression, cell proliferation and malignant transformation. The biological function of chimaerins, however, remains largely unknown. In the second year of my postdoc fellowship, my research work mainly focused on testing our central hypothesis that chimaerin is able to induce cell cycle arrest and inhibit cell proliferation through inactivation of Rac. We found that the mRNA expression levels of beta2-chimaerin in breast cancer cell and tissues are significantly lower than that in normal breast cell and tissues. Expression of beta2-chimaerin impaired the Rac activation by serum and growth factors such as EGF and HRG, reduced cyclin D1 expression and pRb phosphorylation, subsequently induced cell cycle arrest at G1/S phase and inhibited cell proliferation.

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Chimaerin, cell proliferation, cell cycle, breast cancer, signal transduction

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Introduction

β2-Chimaerin is a member of the "non-protein kinase C (PKC)" intracellular receptors for the phorbol ester tumor promoters and the second messenger diacylglycerol (DAG). β2-Chimaerin possesses a C-terminal Rac-GAP (GTPase-activating protein) domain that accelerates the hydrolysis of GTP from the Rac GTPase, leading to its inactivation (Caloca *et al.*, 2003; Canagarajah *et al.*, 2004). Studies have shown that Rac plays a critical role in the control of actin cytoskeleton, cell proliferation, cell cycle progression and malignant transformation (Olson et al., 1995; Qiu et al., 1995; Mettouchi et al., 2001). It has also been reported recently that Rac is overexpressed or hyperactivated in human breast cancer cells (Fritz et al., 1999; Schnelzer et al., 2000). Therefore, the research focus of my second year postdoc fellowship is to test our central hypothesis that by inhibiting Rac function in breast cancer cells, chimaerin will induce cell cycle arrest and impair breast cancer cell proliferation (Specific Aim 2 in my original proposal). As planned in *Task 2* in my original Statement of Work, I have done the following research work in the second year of my postdoc fellowship: (1) comparing chimaerin expression levels between human normal and cancer breast cancer cell lines, and between human normal and cancer breast tissues; (2) determining the Rac-GTP levels in breast cells upon infection with chimaerin-adenoviruses and stimulation with serum or growth factors; (3) examining if chimaerins regulate cell proliferation and cell cycle progression in breast cancer cells; (4) studying the mechanisms of how chimaerins regulate cell proliferation and cell cycle progression in breast cancer cells; and (5) preliminary researches on how chimaerins regulate signaling pathways that control breast cancer cell proliferation (*Task 3*).

Body

In the second year of my fellowship, as originally proposed and outlined in the approved Statement of Work, my researches mainly focus on the effect of chimaerins on breast cancer cell proliferation (*Task 2*). The main findings have been published in *Journal of Biological Chemistry* (2005 July 1; 280: 24363-70) (Please refer to the appended paper).

In addition to studies on *Task 2*, I also performed some preliminary studies on how chimaerins regulate signaling pathways that control breast cancer cell proliferation (*Task 3*). In this part of study, I found that heregulin β1 (HRG), an important EGF-like growth factor for breast carcinogenesis (Falls, 2003), is capable of activating Rac in breast cancer cells (MCF-7 and T-47D cells). As shown in Fig. 1, HRG-induced Rac activation is dose-(Figure 1A) and time-dependent (Figure 1B).

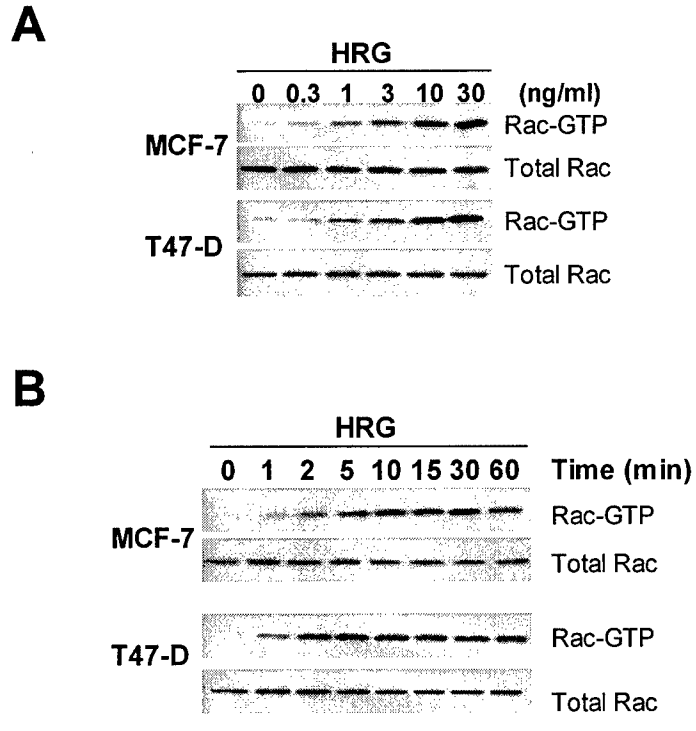

Fig. 1 Heregulin β1 (HRG) activates Rac in breast cancer cells in a does (A)- and time (B)-dependent way. After 48 h serum starvation, cells were treated with HRG as indicated in figures (HRG treatment is 5 min in *panel A* and the concentration of HRG is 10 ng/ml in *panel B* treatment). Rac-GTP levels were measured using a pull-down assay as described in attached paper (*Journal of Biological Chemistry* 2005 July 1; 280: 24363-70).

Moreover, I also found that expression of β2-chimaerin inhibits HRG-induced Rac activation (Figure 2). Inactivation of Rac by β2-chimaerin also impairs HRG-induced mitogen-activated protein kinase (MAPK) activation including Erk1/2 and JNK, important MAPKs for breast cancer cell proliferation by HRG stimulation. These findings were presented at the 96th Annual Meeting of American Association for Cancer Research held on April 16-20, 2005, at Anaheim, CA; and Era of Hope 05 Department of Defense Breast Cancer Research Program Meeting held on June 8-11, 2005, at Philadelphia, PA (please see the appended abstracts).

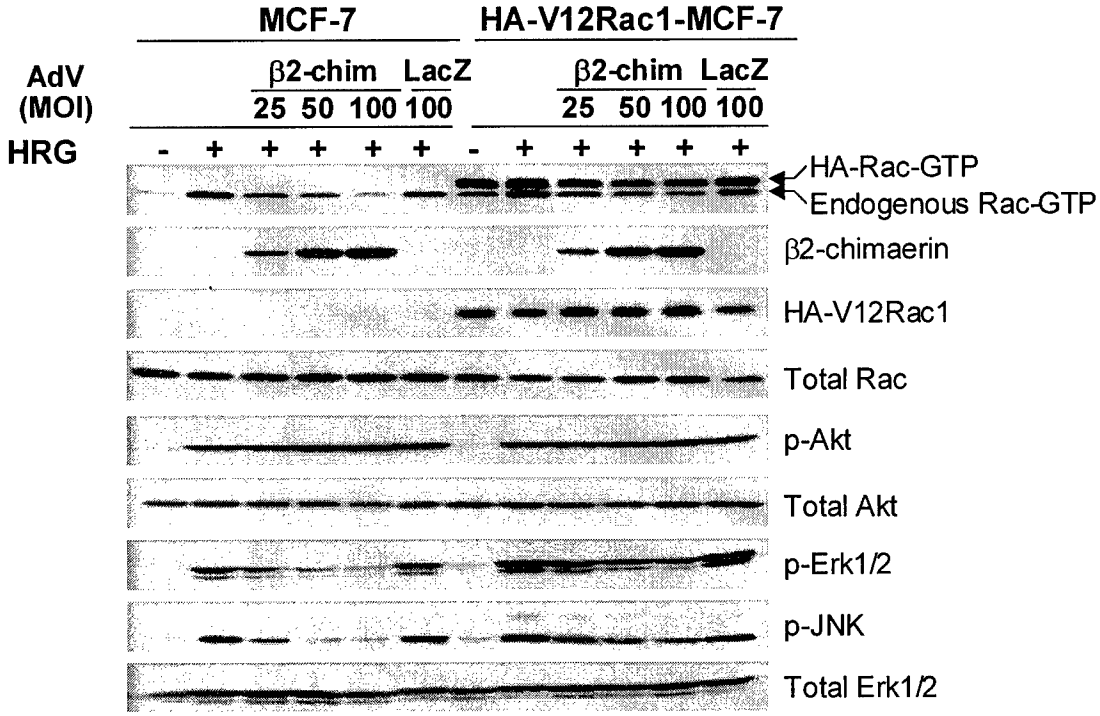

Fig. 2 Inhibition of β2-chimaerin on HRG-induced Erk1/2 and JNK MAPK activation in MCF-7 cells and the reversion by constitutively active Rac (V12Rac1). MCF-7 cells were infected with β2-chimaerin-AdV for 16 h and stimulated with HRG (10 ng/ml) for 10 min. Cells were collected using Tris-SDS buffer and protein concentration in cell lysate was determined using the Bio-Rad protein assay kit. The expression of β2-chimaerin was examined by Western blot; the Rac-GTP levels were determined using a pull-down assay; the phosphorylation of Akt, Erk1/2 and JNK was analyzed by Western blot using specific antibodies as indicated in the figure.

As shown in Figure 2, I also generated a stable MCF-7 cells line that express constitutively active Rac (V12Rac1). Expression of constitutively active Rac (V12Rac1) significantly rescued the inhibitory effect of β2-chimaerin on HRG-induced Erk1/2 and JNK MAPK activation. These results indicate that Rac activation plays an important role in HRG-mediated MAPK activation, and β2-chimaerin may regulate breast cancer cell proliferation through modulating Rac and MAPK activity.

In the second year of my training, under the supervision of my mentor Dr. Kazanietz, I have written and published a research paper entitled "Rac-GAP-

dependent Inhibition of Breast Cancer Cell Proliferation by β2-Chimaerin" in *Journal of Biological Chemistry* (please see the appended paper JBC, 2005, 280: 24363-24370).

Key Research Accomplishments

1. It has been found that the β2-chimaerin mRNA expression levels in breast cancer cell and tissues are significantly lower than that in normal breast cell tissues (please refer to the appended paper JBC, 2005, 280: 24363-24370).

2. Expression of β2-chimaerin inhibits Rac activation in breast cancer cells stimulated by serum, EGF or HRG (please refer to the appended paper JBC, 2005, 280: 24363-24370).

3. Expression of β2-chimaerin or its GAP-domain (β-GAP) induces MCF-7 cell cycle arrest and impairs breast cancer cell proliferation. The inhibition of β2-chimaerin on breast cancer cell growth is dependent on its Rac-GAP activity (please refer to the appended paper JBC, 2005, 280: 24363-24370).

4. Various stable cell lines expressing constitutively active Rac1 (V12Rac1), Cdc42 (V12Cdc42), and RhoA (V14RhoA) have been generated. Moreover, it has been found that expression of constitutively active Rac or Cdc42, but not RhoA, significantly rescued the inhibitory effect of β2-chimaerin on breast cancer cell proliferation (please refer to the appended paper JBC, 2005, 280: 24363-24370).

5. It has also been found that β2-chimaerin inhibits breast cancer cell proliferation through inactivating Rac and decreasing Rb phosphorylation and cyclin D1 level, without affecting the levels of cyclin A, E , and CdK2 and 4 (please refer to the appended paper JBC, 2005, 280: 24363-24370).

6. Preliminary studies suggest that β2-chimaerin may inhibit breast cancer cell proliferation through modulating Rac-mediated Erk1/2 and JNK MAPK activation (please refer to the results shown above).

Reportable Outcomes

1. One published research paper:

   Yang C, Liu Y, Leskow FC, Weaver VM, Kazanietz MG MG. Rac-GAP-dependent inhibition of breast cancer cell proliferation by β2-chimerin. *Journal of Biological Chemistry* 2005 July 1; 280(26): 24363-24370.

2. Two conference poster presentations:

   (1). **Yang C,** Liu Y, and Kazanietz MG. Essential role for Rac1 in heregulin β1-induced mitogenic signaling in human breast cancer cells. *Proceedings of the 96th Annual Meeting of American Association for Cancer Research*, p868, April 16-20, 2005. Anaheim, CA.

   (2). **Yang C,** Liu Y, and Kazanietz MG. Heregulin β1-induced Rac activation promotes breast cancer cell proliferation. *Proceedings of Era of Hope-05 Department of Defense Breast Cancer Research Program Meeting*, p223, June 8-11, 2005. Philadelphia, PA.

Conclusions

Based on the results obtained from my second year study, I can make the following conclusions: (1) the mRNA expression level of β2-chimaerin, a specific Rac-GAP and one of the most widely-expressed chimaerin isoforms, is much lower in breast cancer cell and tissues than that in normal breast cell and tissues; (2) β2-chimaerin inhibits breast cancer cell proliferation through its Rac-GAP activity; and (3) preliminary data indicate that β2-chimaerin is able to inhibit MAPK signaling through inactivation of Rac. These findings suggest that β2-chimaerin might act as a tumor suppressor through modulating the small GTPase Rac activity and provide further rationale for interfering with Rac signaling in breast cancer treatment.

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Abbreviations

AdV, adenovirus;
EGF, epidermal growth factor;
Erk1/2, extracellular signal-regulated kinase1/2;
GAP, GTPase Activating Protein;
HA, hemaglutinin;
HRG, heregulin β1;
JNK, c-Jun N-terminal kinase;
MAPK, mitogen-activated protein kinase;
MOI, multiplicities of infection;

Appendices

1. **Yang C**, Liu Y, Leskow FC, Weaver VM, Kazanietz MG MG. Rac-GAP-dependent inhibition of breast cancer cell proliferation by β2-chimerin. Journal of Biological Chemistry 2005 July 1; 280(26): 24363-24370.

2. **Yang C,** Liu Y, and Kazanietz MG. Essential role for Rac1 in heregulin β1-induced mitogenic signaling in human breast cancer cells. *Proceedings of the 96th Annual Meeting of American Association for Cancer Research*, p868, April 16-20, 2005. Anaheim, CA.

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Rac-GAP-dependent Inhibition of Breast Cancer Cell Proliferation by β2-Chimerin*

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β2-Chimerin is a member of the "non-protein kinase C" intracellular receptors for the second messenger diacylglycerol and the phorbol esters that is yet poorly characterized, particularly in the context of signaling pathways involved in proliferation and cancer progression. β2-Chimerin possesses a C-terminal Rac-GAP (GTPase-activating protein) domain that accelerates the hydrolysis of GTP from the Rac GTPase, leading to its inactivation. We found that β2-chimerin messenger levels are significantly down-regulated in human breast cancer cell lines as well as in breast tumors. Adenoviral delivery of β2-chimerin into MCF-7 breast cancer cells leads to inhibition of proliferation and G_1 cell cycle arrest. Mechanistic studies show that the effect involves the reduction in Rac-GTP levels, cyclin D1 expression, and retinoblastoma dephosphorylation. Studies using the mutated forms of β2-chimerin revealed that these effects were entirely dependent on its C-terminal GAP domain and Rac-GAP activity. Moreover, MCF-7 cells stably expressing active Rac (V12Rac1) but not RhoA (V14RhoA) were insensitive to β2-chimerin-induced inhibition of proliferation and cell cycle progression. The modulation of G_1/S progression by β2-chimerin not only implies an essential role for Rac in breast cancer cell proliferation but also raises the intriguing possibility that diacylglycerol-regulated non-protein kinase C pathways can negatively impact proliferation mechanisms controlled by Rho GTPases.

Chimerins represent a family of four closely related GAPs[1] (GTPase-activating proteins) for small GTPases that were originally characterized as high affinity intracellular receptors for the second messenger diacylglycerol (DAG) and the phorbol ester tumor promoters (1–4). Structurally, chimerins possess a C1 domain highly homologous to those of PKC isozymes (the DAG/phorbol ester binding site) and a C-terminal GAP domain. The α2- and β2-chimerins also have a N-terminal Src homology 2 domain of unknown function, which is not present in the splice variants α1-(or *n*-) and β1-chimerins (5, 6). Very little information is available regarding the regulation, expression, and function of β2-chimerin or the other chimerin isoforms as well as their role in proliferation mechanisms and cancer progression. We have been focusing our attention on β2-chimerin, because there is emerging evidence that this isoform is directly regulated by phorbol esters (4, 7) as well as tyrosine-kinase receptors that couple to DAG generation.[2] Importantly, early studies in gliomas have suggested a potential role for β2-chimerin as a tumor suppressor (8) but its relevance in other cancer models is still unknown.

In vitro studies have shown that the C-terminal domain of chimerins is capable of accelerating GTP hydrolysis from the small GTPase Rac1 without affecting the activity of RhoA or Cdc42 GTPases (9, 10). Our recent studies in COS cells revealed that β2-chimerin decreases cellular Rac-GTP levels and inhibits the elevation of Rac-GTP levels caused by epidermal growth factor (EGF) (10, 11). Rac GTPase is known to act as a molecular switch, cycling between an active GTP-bound state (Rac-GTP) and an inactive GDP-bound state (Rac-GDP). This switch is regulated by three groups of molecules: 1) guanine nucleotide exchange factors, such as Vav and Tiam-1, that promote its conversion to the active GTP-bound form; 2) guanine nucleotide dissociation inhibitors; and 3) GAPs, which stimulate intrinsic GTPase activity, thus leading to Rac inactivation (12, 13). Active Rac interacts with various effectors to initiate downstream signaling events that control the dynamics of actin cytoskeleton reorganization, migration, adhesion, and gene expression (14–17). Rac and Rac-guanine nucleotide exchange factors play key roles in the control of various aspects of malignant transformation and the metastatic cascade in various models, including breast cancer cells (18–20). Several laboratories (21–25) have proposed a role for Rac in the control of mitogenesis through its ability to regulate G_1/S transition and cyclin D1 expression. Moreover, Rac and other members of the Rho GTPase family are overexpressed in human tumors such as in breast cancer (26, 27) and hyperactivation of Rac leading to a higher rate of cell proliferation has been found in cellular models of human breast cancer (28). Targeted expression of an activated Rac mutant in mammary epithelium causes mam-

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[1] The abbreviations used are: GAP, GTPase-activating protein; AdV, adenovirus; BrdUrd, 5-bromo-2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; DAG, diacylglycerol; EGF, epidermal growth factor; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PBD, p21 binding domain; PKC, protein kinase C; IDC, infiltrating ductal carcinoma; Q-PCR, quantitative real-time PCR; D1-RetroV, D1 T286A retrovirus; PBS, phosphate-buffered saline; Rb, retinoblastoma; m.o.i., multiplicity of infection(s); ANOVA, analysis of variance.

[2] H. Wang, C. Yang, F. Coluccio Leskow, J. Sun, B. Canagarajah, J. H. Hurley, and M. G. Kazanietz, submitted for publication.

mary gland lesions (29). In addition, there is strong evidence that Rac effectors such as p21-activated kinase 1 are dysregulated in breast cancer cells (30). Collectively, these findings suggest critical implications of Rac in tumorigenesis, particularly in models of breast cancer.

In this paper, we investigated the expression of the DAG/phorbol ester receptor β2-chimerin in breast cancer and its role in proliferation. We have found that β2-chimerin mRNA levels are strikingly reduced in breast cancer cell lines and tissues. By means of adenoviral delivery into MCF-7 breast cancer cells, we have found that β2-chimerin, but not the mutated forms lacking Rac-GAP activity, causes a significant impairment in G_1/S cell cycle progression due to a reduction in the expression levels of cyclin D1. The effect of β2-chimerin is strictly dependent on its ability to inhibit Rac function via the C-terminal GAP domain, suggesting the possibility that Rac-mediated control of cell proliferation is modulated by DAG-regulated pathways.

EXPERIMENTAL PROCEDURES

Human Breast Non-malignant and Cancer Cell Lines—Human breast cancer cell lines MCF-7, T-47D, MDA-MB231, MDA-MB-435, MDA-MB-468, Hs578T, and human breast immortalized non-malignant MCF-10A cells were purchased from ATCC and cultured as recommended by the provider. The human non-malignant breast cell line HMT-3522 and its malignant derivative T4-2 were cultured as previously described (31). MCF-7-Tet-On cells were purchased from Clontech and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml G418.

Examination of β2-Chimerin mRNA Levels in Human Breast Cells and Tissues—For tissue RNA, 10 pairs of high quality human breast cancer tissue total RNA and matched-normal tissue total RNA (from the same patient) were purchased from Clinomix Biosciences, Inc. (Watervliet, NY). All 10 patients were diagnosed as infiltrating ductal carcinoma (IDC) at different stages including two Stage I IDCs (samples 1–2), two Stage II IDCs (samples 3–4), two Stage III IDCs (samples 5–6), and four Stage IV IDCs (samples 7–10). Total RNA was prepared using TRIzol and reversibly transcripted using SuperScript™ II Reverse Transcriptase (Invitrogen). β2-Chimerin mRNA levels were determined either by standard PCR (30 cycles) using the following primers: 5′-TGATCTCAAGAGGATCAAGAA-3′ (forward) and 5′-TTGGAATAGGTATCATATGTG-3′ (reverse), which specifically amplify a 297-bp fragment of β2-chimerin. Primers used for real-time PCR (Q-PCR) are described elsewhere (32). The real-time PCR reactions were plated in triplicate and performed in 384-well plates using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization (32).

Generation of Adenoviruses (AdVs)—AdVs were generated with the AdEasy™ adenoviral vector system (Stratagene). Generation of the β2-chimerin and β-GAP AdVs was described elsewhere (10, 32). For the generation of ΔEIE-β2-chimerin adenoviral construct, a XhoI-MluI insert comprising the mutant ΔEIE-β2-chimerin (10) was ligated into pShuttle-CMV-HA. A similar strategy was used for the generation of an AdV for the mutant I130A-β2-chimerin (33). A control LacZ-AdV was generated from pShuttle-CMV-LacZ (provided by the kit) and therefore has the same backbone as the β2-chimerin AdVs. For adenoviral infections, MCF-7 cells in 6-well plates growing in serum-free DMEM were infected with various AdVs for 16 h. AdVs were removed after extensive washing, and experiments were performed 48 h later.

Generation of Stable Cell Lines Expressing Constitutively Active Small GTPases—Stable cell lines expressing active mutants of Rac1, Cdc42, or RhoA were generated upon transfection of MCF-7-Tet-On cells using FuGENE 6 (Roche Applied Science) followed by G418/hygromycin selection. The following plasmids were used: pTRE-HA (empty vector); pTRE-HA-V12Cdc42 (a kind gift from Dr. Margaret Chou, University of Pennsylvania); pTRE-HA-V12Rac1; and pTRE-HA-V14RhoA. These last two plasmids were generated by subcloning V12Rac1 (isolated from pcDNA3-V12Rac1) and V14RhoA (isolated from pXDR-HA-V14RhoA) into pTRE-HA, respectively. pcDNA3-V12 Rac1 and pXDR-HA-V14RhoA were generous gifts from Dr. Rick Assoian (University of Pennsylvania).

Generation of Cyclin D1 T286A Retrovirus (D1-RetroV)—D1-RetroV was generated by co-transfecting pMX-FLAG-D1 T286A (34) (a cyclin D1 mutant resistant to proteolysis degradation, 10 μg) and a helper plasmid (p-Helper, 5 μg) into 293T packaging cells (generous gifts from Dr. J. A. Diehl, University of Pennsylvania). Co-transfection was performed in a 10-cm dish (5×10^6 cells/dish) in DMEM supplemented with 10% FBS using Lipofectamine Plus (Invitrogen) following the instructions from the manufacturer. The medium was collected 48 h later, centrifuged ($1000 \times g$, 5 min), and filtered with a 0.24-μm filter. A control retrovirus using pMX empty vector was also generated (V-RetroV). For retroviral infections, MCF-7 cells cultured in 6-cm dishes were infected with 3 ml of either D1-RetroV or V-RetroV in the presence of Polybrene (10 μg/ml, Sigma) for 24 h.

Cell Proliferation and Cell Cycle Analysis—Cell proliferation was assessed by BrdUrd incorporation and by the MTS assay (CellTiter 96® Aqueous One solution cell proliferation assay, Promega). After overnight infection (16 h) with the different AdVs, cells were washed once with PBS and incubated in serum-free DMEM for 24 h and then cultured in DMEM supplemented with 10% FBS for another 24 h. BrdUrd (Sigma) was then added into the medium for 30 min (final concentration: 0.2 mM). Cells were then collected by trypsinization, washed with PBS, and fixed with 70% ethanol for BrdUrd incorporation analysis using flow cytometry (35). For the MTS assay, after overnight adenoviral infection in 10-cm dishes, cells were collected using trypsin, counted, and then seeded onto 96-well plates (1×10^4 cells/well in 100 μl of DMEM supplemented with 10% FBS). MTS was added after 24, 48, or 72 h, and absorbance was measured at 490 nm (36). For cell cycle analysis, after overnight (16 h) adenoviral infection, cells were washed once with PBS, incubated in serum-free DMEM for 24 h, and then cultured in DMEM supplemented with 10% FBS for another 24 h. Cells were then collected by trypsinization and analyzed using flow cytometry as previously described (37).

Western Blot—15 μg of proteins were used for Western blot analysis (38). The following antibodies were used: anti-β2-chimerin (10, 11); anti-Rac; anti-Cdc42; anti-cyclins A, D1, and E (Upstate Biotechnology); anti-pRb (BD Transduction Laboratories); anti-HA tag (Cell Signaling); and anti-β-actin (Sigma).

Rac-GTP and Cdc42-GTP Pull-down Assays—After overnight infection (16 h) with different AdVs, cells were incubated in serum-free DMEM for 24 h and then stimulated with EGF (100 ng/ml, 1 min). Alternatively, after adenoviral infection, cells were cultured in 10% FBS DMEM for an additional 24-h period. Rac-GTP and Cdc42-GTP levels were determined with a "pull-down" assay using the PBD (p21-binding domain) of p21-activated kinase, as previously described (10, 11), and using either anti-Rac or anti-Cdc42 antibodies for detection, respectively.

Statistical Analysis—Data were analyzed using either a Student's *t* test or one-way analysis of variance (ANOVA) with Scheffe's test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Reduced Expression of β2-Chimerin in Human Breast Cancer Cells and Tissues—The expression of β2-chimerin in normal and breast cancer cells is unknown. Using standard PCR analysis, we found high levels of β2-chimerin mRNA in non-malignant immortalized MCF-10A cells. On the other hand, in all of the cancer cell lines examined, the β2-chimerin transcript was barely detected or dramatically reduced (Fig. 1*A*). The results were confirmed by a quantitative analysis using Q-PCR. Indeed, β2-chimerin mRNA was not detected in MCF-7 and Hs578T cells and it was very low in T-47D, MDA-MB-231, and MDA-MB-435 cells. Only MDA-MB-468 cells showed significant levels of β2-chimerin transcript, although much lower than MCF-10A cells (Fig. 1*B*). Similarly, whereas β2-chimerin mRNA was readily detected in the non-malignant breast cell line HMT3522 (31), it was barely detectable in its malignant derivative (T4-2) (Fig. 1, *A* and *B*). We next examined β2-chimerin mRNA levels in a small sample of human breast cancer tissues and their corresponding matched-normal tissues (from the same patient). It was found that the expression of β2-chimerin mRNA in normal tissues was highly variable. However, among the 10 patients, the β2-chimerin transcript was significantly lower in the cancer tissues of 7 patients (Fig. 1*C*). Together, these results reveal a significant reduction of β2-chimerin expression in human breast cancer.

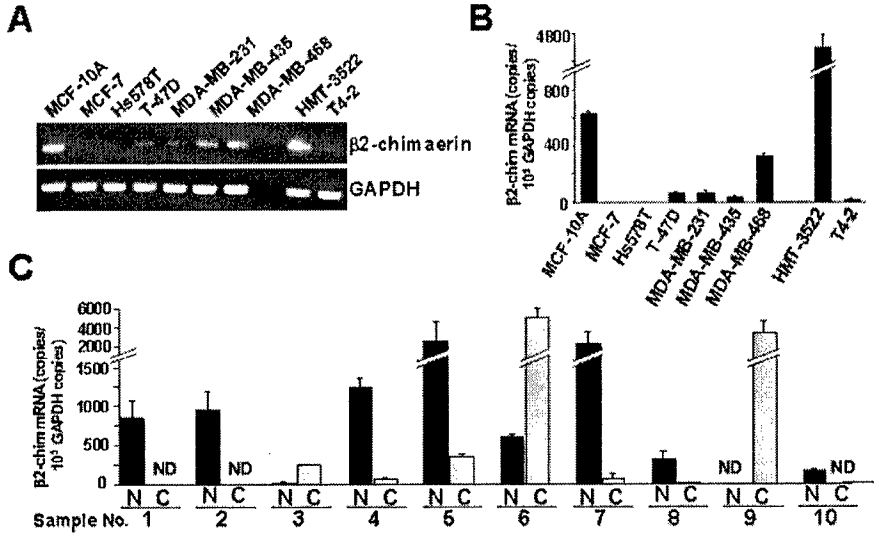

FIG. 1. **β2-Chimerin transcript levels in human breast cancer cells and tissues.** *Panel A*, β2-chimerin mRNA levels in non-malignant and cancer human breast cell lines, as determined by PCR. A representative experiment is shown. Similar results were obtained in two additional experiments. *Panel B*, analysis of β2-chimerin mRNA levels in non-malignant and cancer human breast cell lines using Q-PCR. *Panel C*, Q-PCR analysis of β2-chimerin mRNA levels in 10 pairs of human breast cancer tissue RNA (*C*) and corresponding matched normal breast tissue RNA (*N*). Q-PCR results are presented as mean ± S.D. (n = 3). *ND*, not detected.

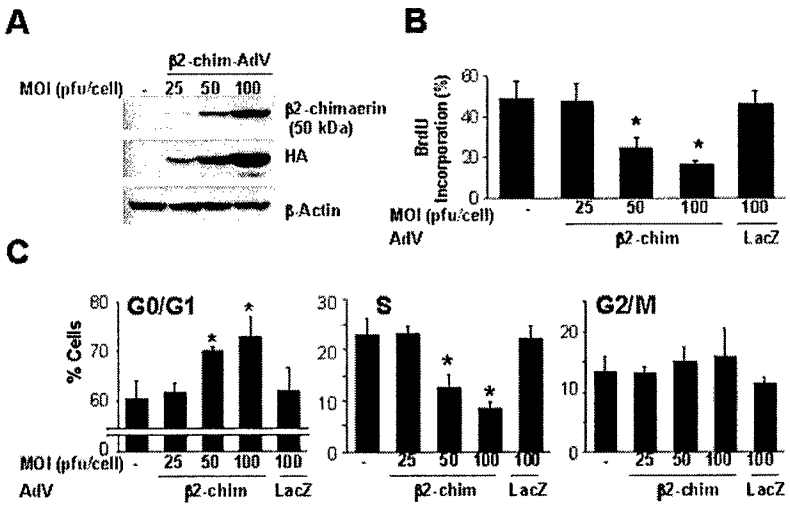

FIG. 2. **β2-Chimerin inhibits BrdUrd incorporation and G_1/S transition in MCF-7 cells.** *Panel A*, MCF-7 cells were infected with different m.o.i. of β2-chimerin-AdV or LacZ-AdV, as indicated in the figure, and the expression of β2-chimerin was assessed by Western blot 48 h after infection using either anti-HA or anti-β2-chimerin antibodies. *Panel B*, BrdUrd incorporation was determined in cells infected with either β2-chimerin-AdV or LacZ-AdV using flow cytometry, as described under "Experimental Procedures." *Panel C*, MCF-7 cells were infected with the β2-chimerin-AdV at different m.o.i. Cell cycle analysis was carried out 48 h later using flow cytometry. Data are presented as mean ± S.D. (n = 3). *, $p < 0.05$ compared with control cells.

Ectopic Expression of β2-Chimerin Inhibits Proliferation of MCF-7 Cells—Because Rac is known to control proliferation and there is evidence for Rac hyperactivation in breast cancer models, we examined how β2-chimerin affects human breast cancer cell proliferation. HA-tagged β2-chimerin was introduced into MCF-7 cells using an adenoviral gene delivery approach. Upon infection of MCF-7 cells with different m.o.i. of the β2-chimerin-AdV, HA-β2-chimerin was readily detected (Fig. 2*A*). Interestingly, β2-chimerin dose-dependently reduced BrdUrd incorporation in MCF-7 cells. On the other hand, infection with a control AdV (LacZ-AdV, 100 m.o.i.) did not change BrdUrd incorporation significantly (Fig. 2*B*).

To begin elucidating the mechanisms involved in the β2-chimerin effect, we examined cell cycle progression (Fig. 2*C*). Flow cytometry analysis revealed a significantly higher percentage of cells in G_0/G_1 phase upon infection with the β2-chimerin-AdV compared with control (non-infected) or LacZ-AdV-infected MCF-7 cells. The effect was proportional to the m.o.i. used for infection. A concomitant decrease in the percentage of cells in S phase was observed, but no significant changes were observed in the number of cells in G_2/M phase.

The Anti-proliferative Effect of β2-Chimerin Is Dependent on a Functional β-GAP Domain—To examine whether the inhibitory effect on cell proliferation depends on β2-chimerin Rac-GAP activity, we expressed the C-terminal catalytic region of β2-chimerin (β-GAP domain) using an AdV. Infection of MCF-7 cells with β-GAP-AdV resulted in a m.o.i.-dependent increase in the expression of the β-GAP domain (Fig. 3*A*). Expression of β-GAP caused similar effects on cell proliferation and cell cycle progression as those observed with full-length β2-chimerin (Fig. 3, *B* and *C*). We then took advantage of the β2-chimerin mutant ΔEIE-β2-chimerin (deletion in positions 298–300 in the β-GAP domain), which is unable to accelerate GTP hydrolysis from Rac (10). An AdV for the GAP-inactive ΔEIE-β2-chimerin was generated. Upon delivery into MCF-7 cells, ΔEIE-β2-chimerin-AdV was unable to inhibit BrdUrd incorporation (Fig. 3, *D* and *E*).

β2-Chimerin Reduces Cyclin D1 Expression and Inhibits pRb Phosphorylation—Because expression of β2-chimerin in MCF-7 cells leads to G_1/S arrest, we next assessed the effect of β2-chimerin on pRb phosphorylation. The expression of β2-chimerin dose-dependently reduced pRb phosphorylation (Fig. 4*A*). Notably, β2-chimerin significantly inhibited the expression of cyclin D1. β2-Chimerin also reduced the expression of cyclin A and caused a slight increase in cyclin E levels. On the other hand, cells infected with control LacZ-AdV (100 m.o.i.) showed no obvious alterations in cyclin expression and pRb phosphorylation. Infection of MCF-7 cells with β2-chimerin-AdV did not cause any significant changes on the expression of cyclin-dependent kinases 2, 4, and 6 (data not shown). Infection of MCF-7 cells with the β-GAP-AdV also led to a reduction in cyclin D1 levels and pRb phosphorylation (Fig. 4*A*). However,

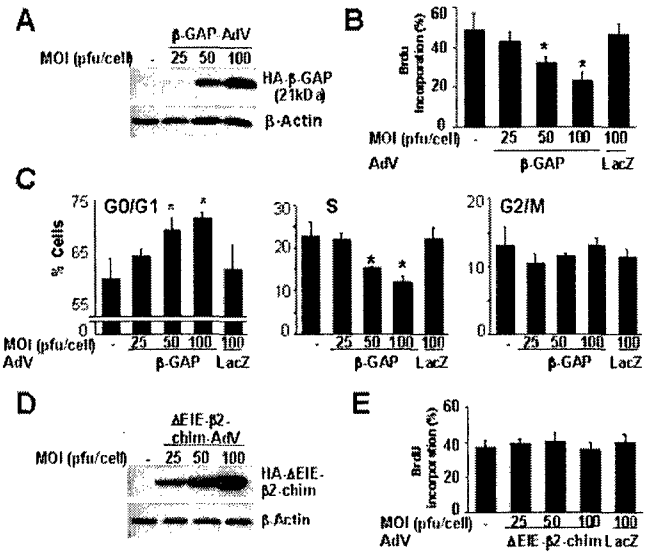

FIG. 3. **The anti-proliferative effect of β2-chimerin depends on a functional β-GAP domain.** Expression of β-GAP or ΔEIE-β2-chimerin was determined by Western blot 48 h after infection of MCF-7 cells with increasing m.o.i. of either β-GAP-AdV (*panel A*) or ΔEIE-β2-chimerin-AdV (*panel D*). *Panels B* and *E*, BrdUrd incorporation was determined in cells infected with either β-GAP-AdV (*panel B*) or ΔEIE-β2-chimerin-AdV (*panel E*) using flow cytometry, as described under "Experimental Procedures." *Panel C*, MCF-7 cells were infected with the β-GAP-AdV at different m.o.i. Cell cycle analysis was determined 48 h later using flow cytometry. Data are presented as the mean ± S.D. ($n = 3$). *, $p < 0.05$ compared with control cells.

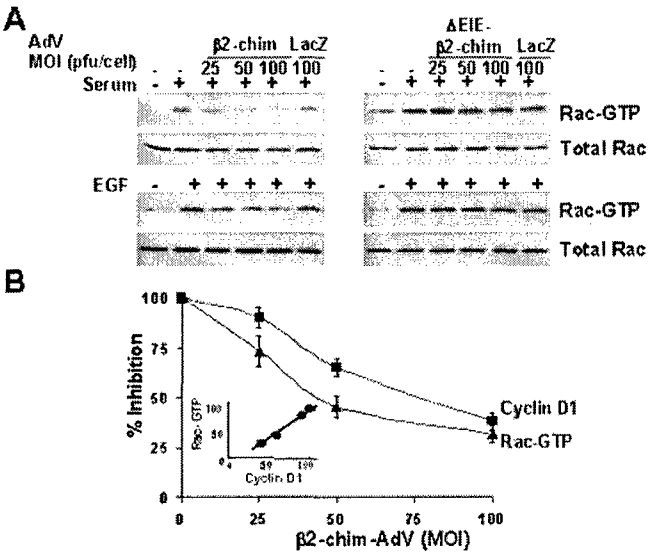

FIG. 5. **Effect of β2-chimerin on Rac-GTP levels in MCF-7 cells.** *Panel A*, cells were infected with different AdVs at the m.o.i. indicated in the figure. After serum starvation (24 h), cells were either incubated with 10% FBS DMEM for 24 h or stimulated with EGF (100 ng/ml, 1 min). Rac-GTP levels were determined using a PBD pull-down assay, as described under "Experimental Procedures." *Panel B*, densitometric analysis of inhibitory effect of β2-chimerin on serum-induced Rac activation normalized to the corresponding total Rac levels in each case. The panel also shows the densitometric analysis of cyclin D1 inhibition by the β2-chimerin-AdV from Fig. 4. *Inset* shows the correlation between the inhibition of Rac-GTP and cyclin D1 levels at the different m.o.i. of the β2-chimerin-AdV (expressed as percentage of control cells).

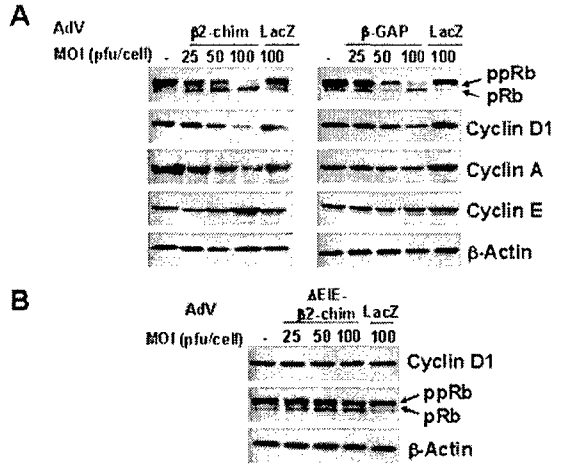

FIG. 4. **β2-Chimerin reduces cyclin D1 expression and inhibits its pRb phosphorylation.** MCF-7 cells were infected with increasing m.o.i. of AdVs for β2-chimerin, β-GAP (*panel A*), or ΔEIE-β2-chimerin (*panel B*). A LacZ-AdV (100 m.o.i.) was used as a control. After 48 h, cell extracts were prepared and subjected to Western blot analysis for pRB and cyclins. Similar results were observed in three independent experiments.

the Rac-GAP inactive mutant, ΔEIE-β2-chimerin, did not impair cyclin D1 expression or pRb phosphorylation (Fig. 4*B*). Taken together, these results suggest that the inhibition of G_1/S transition by β2-chimerin via its β-GAP domain involves the reduction of cyclin D1 and pRb phosphorylation levels.

Inhibition of Rac by β2-Chimerin in MCF-7 Cells—β2-Chimerin has specificity for the Rac GTPase both in *in vitro* GAP assays and in COS-1 cells but does not affect RhoA or Cdc42 activity (10, 11). EGF (100 ng/ml, 1 min) caused a 3.3 ± 0.5-fold ($n = 3$) increase in Rac-GTP levels in MCF-7 cells, which was significantly impaired by the expression of β2 chimerin. β2-Chimerin also reduced Rac-GTP levels in MCF-7 cells growing in 10% serum. The effect was proportional to the m.o.i. used for infection, and it was not observed with the GAP-inactive mutant, ΔEIE-β2-chimerin (Fig. 5*A*). A densitometric analysis of the β2-chimerin effect on serum-induced activation of Rac is presented in Fig. 5*B*. A striking correlation was observed between the inhibitory effect of β2-chimerin on Rac activity and the reduction in cyclin D1 levels by different m.o.i. of the β2-chimerin-AdV ($r = 0.95$).

Ectopic Expression of Cyclin D1 Rescues the Anti-proliferative Effect of β2-Chimerin—To further explore the link between Rac and cyclin D1 in our experimental model, we expressed cyclin D1 using a retroviral approach (Fig. 6*A*). Interestingly, the ectopic expression of cyclin D1 using the D1-RetroV significantly rescued the anti-proliferative effect of β2-chimerin, whereas the control retrovirus (V-RetroV) did not (Fig. 6*B*).

MCF-7 Cells Expressing Constitutively Active Rac1 Are Insensitive to β2-Chimerin—We reasoned that the expression of a constitutively active Rac mutant in MCF-7 cells should impair the effects of β2-chimerin on cell proliferation and cell cycle progression. A MCF-7 cell line stably expressing active V12Rac1 was generated (HA-V12Rac1-MCF-7) (Fig. 7*A*). These cells show higher levels of Rac-GTP than control (vector-transfected) cells (Fig. 7*D*). Interestingly, whereas β2-chimerin markedly reduced proliferation in control MCF-7 cells, HA-V12Rac1-MCF-7 cells were insensitive to β2-chimerin (Fig. 7*B*). Consistent with these results, adenoviral delivery of β2-chimerin into HA-V12Rac1-MCF-7 cells did not reduce cyclin D1 levels or cause pRb dephosphorylation (Fig. 7*A*).

We then determined whether the expression of other active Rho-GTPases could rescue the effect of β2-chimerin. MCF-7 cell lines stably expressing constitutively active Cdc42 (V12Cdc42) or RhoA (V14RhoA) were generated (Fig. 7*A*). Similar to control MCF-7 cells, HA-V14RhoA-MCF-7 cells were highly sensitive to β2-chimerin for the inhibition of cell proliferation, reduction of cyclin D1, and Rb dephosphorylation (Fig. 7, *A* and *B*). Unexpectedly, in cells expressing active Cdc42,

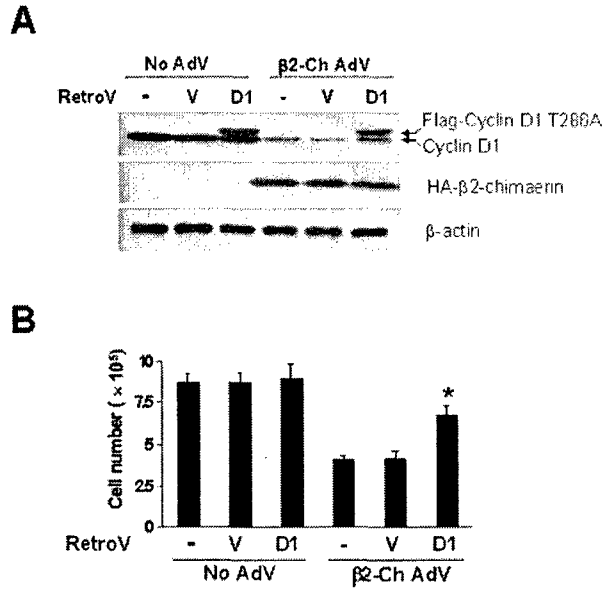

FIG. 6. **Ectopic expression of cyclin D1 rescues the anti-proliferative effect of β2-chimerin.** MCF-7 cells were infected with either a FLAG-cyclin D1 T286A retrovirus (D1-RetroV) (*D1*) or a control retrovirus (V-RetroV) (*V*) for 24 h. After extensive washings with PBS, cells were serum-starved for 8 h and then infected with HA-β2-chimerin-AdV (100 m.o.i.) for 16 h. After 24-h serum starvation, cells were stimulated with 10% FBS for 24 h. The expression of cyclin D1 and HA-β2-chimerin was determined by Western blot (*panel A*). Cell proliferation was determined by hemacytometer counting (*panel B*). Data are presented as mean ± S.D. ($n = 3$). *, $p < 0.05$ compared with cells infected with HA-β2-chimerin-AdV without retroviral infection or with control retroviral infection.

adenoviral delivery of β2-chimerin was unable to inhibit cell proliferation, cyclin D1 expression, and pRb phosphorylation (Fig. 7, *A* and *B*). To further examine the mechanisms involved in the protective effect of V12Cdc42, we determined Cdc42-GTP levels in response to EGF (100 μg/ml, 1 min). A 3.1 ± 0.6-fold ($n = 3$) increase in Cdc42-GTP levels was observed in response to the growth factor, which was not affected by the β2-chimerin-AdV, even at the highest m.o.i. used (100 plaque-forming units/cell) (Fig. 7*C*). Interestingly, we found that basal Rac-GTP levels were elevated in HA-V12Cdc42-MCF-7 cells (Fig. 7, *D* and *E*), which probably explain the protective effect of active Cdc42 on β2-chimerin-induced inhibition of cyclin D1 levels, pRb phosphorylation, and cell proliferation.

A Hyperactive β2-Chimerin Mutant Is a Potent Inhibitor of Cyclin D1 Expression and Proliferation—Based on structural predictions gained from the recently solved structure of β2-chimerin, we generated an AdV encoding for a β2-chimerin mutant locked in the constitutively active conformation. This mutant, I130A-β2-chimerin, was shown to have constitutive Rac-GAP activity when expressed in COS-1 cells by bypassing lipid activation (33). An AdV encoding for I130A-β2-chimerin was generated and used to infect MCF-7 cells. We optimized conditions to achieve similar low levels of expression as those observed in the non-malignant breast cell line HMT3522 cells (as detected by Q-PCR, Table I). In this case, we used a lower m.o.i. and shorter expression times (16 h instead of 40 h) and the levels of the mutant I130A-β2-chimerin in MCF-7 cells were well below the detection levels using Western blot. Under these experimental conditions, the wild-type β2-chimerin still showed very high levels of expression by Western blot and caused a ~25% reduction in Rac-GTP levels (Fig. 8*A*). Remark-

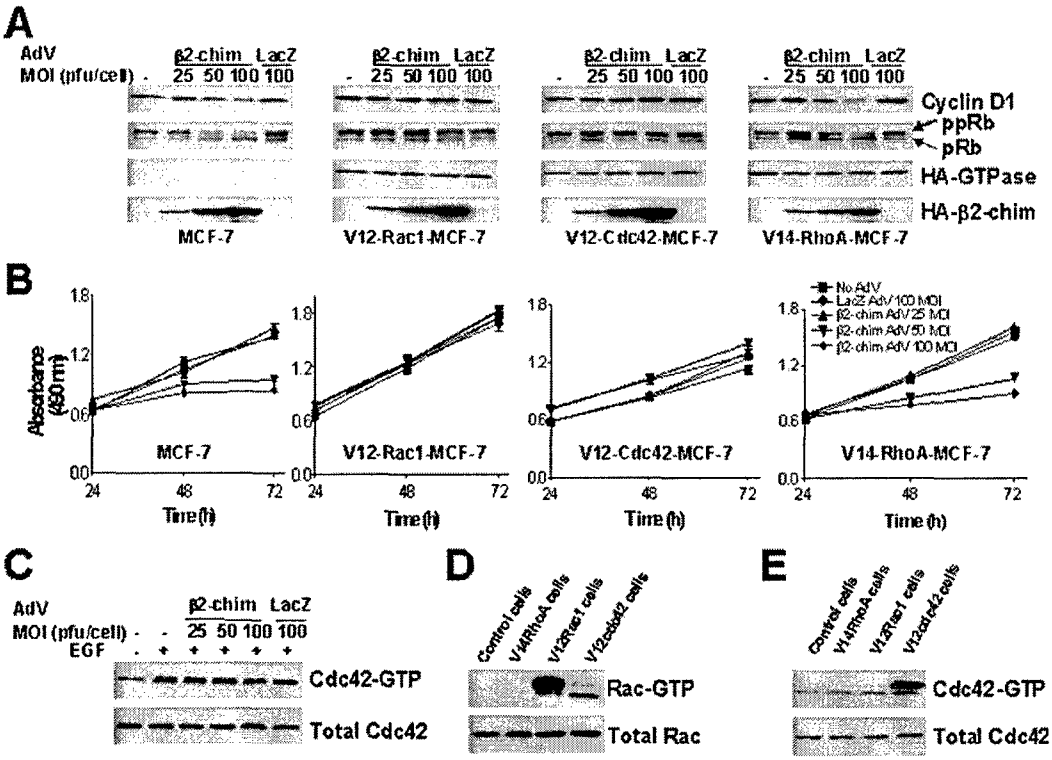

FIG. 7. **Effect of β2-chimerin in MCF-7 cells stably expressing constitutively active GTPases.** *Panel A*, generation of MCF-7 cell lines expressing HA-tagged V12Rac1, V12Cdc42, and V14RhoA. Western blots were performed in cell lines stably expressing active GTPases and control cells (transfected with empty vector) that had been infected with the β2-chimerin AdV for 48 h. *Panel B*, analysis of cell proliferation in the various cell lines using an MTS assay, as described under "Experimental Procedures." Data are presented as mean ± S.D. ($n = 8$). ■, control (no AdV); ●, LacZ-AdV (100 m.o.i.); ▲, β2-chimerin-AdV (25 m.o.i.); ▼, β2-chimerin-AdV (50 m.o.i.); ♦, β2-chimerin-AdV (100 m.o.i.). *Panel C*, lack of effect of β2-chimerin on Cdc42 activity. Control MCF-7 cells were infected with different m.o.i. of β2-chimerin-AdV, and upon serum starvation for 24 h, they were treated with EGF (100 ng/ml, 1 min). Cdc42-GTP levels were measured using a pull-down assay. *Panels D* and *E*, determination of Rac-GTP and Cdc42-GTP levels in MCF-7 cell lines growing in 10% FBS DMEM. Similar results were obtained in three independent experiments.

ably, even if I130A-β2-chimerin was expressed at very low levels, it caused a 51% reduction in Rac-GTP levels (Fig. 8*A*). Moreover, I130A-β2-chimerin markedly reduced cyclin D1 levels (Fig. 8*B*) and impaired cell proliferation (Fig. 8*C*).

DISCUSSION

Understanding the functional properties of Rac-GAPs is relevant, because Rac is a key player in the process of malignant transformation and metastasis (12, 18, 30). The two most relevant findings in the present study are that β2-chimerin expression is down-regulated in breast cancer and that the expression of this Rac-GAP in MCF-7 breast cancer cells impairs G_1/S cell cycle progression by reducing cyclin D1 levels and Rb phosphorylation. Inhibition of proliferation by β2-chimerin in MCF-7 cells is dependent on the β2-chimerin GAP activity, and indeed, a functionally active GAP domain is required for the anti-mitogenic effect. Our results suggest that Rac activity is critical for G_1/S progression in breast cancer MCF-7 cells.

Analysis of β2-chimerin mRNA levels revealed that breast cancer cells have significantly lower levels than non-malignant cells. This effect is particularly striking when we compare the non-malignant HMT3522 cell line with its malignant derivate T4-2 cell line. Moreover, studies using matched pairs of RNA samples from breast cancer patients revealed that β2-chimerin is significantly down-regulated in 70% of tumor samples. Members of the Rho GTPase family such as Rac and Rho are overexpressed in human tumors, particularly in breast cancer (26, 27). Rac activity was found to be elevated in transformed cells, as recently described in v-Src-transformed fibroblasts, and inhibition of Rac function using dominant-negative Rac mutants dramatically reduced the ability of v-*src* to transform NIH 3T3 cells (39). Small GTPase hyperactivation may be the consequence of enhanced upstream inputs and/or reduced activity of GAPs, as suggested by Mira and co-workers (28) in breast cancer cell models. Various mechanisms can account for the elevated upstream inputs including receptor hyperactivation and/or enhanced activation of Rac-guanine nucleotide exchange factors, such as Tiam1 and Vav (20, 40). On the other hand, the relative contribution of the down-regulation of Rho GAPs in cancer progression and their potential roles as tumor suppressors has not been extensively studied. For example, a recent study (41) has found that the Rho/Cdc42 GAP DLC2 is

TABLE I

Comparison of β2-chimerin mRNA levels in breast cells

The β2-chimerin mRNA levels were determined by Q-PCR as described under "Experimental Procedures." For I130A-β2-chimerin adenoviral infection, MCF-7 cells were serum-starved for 8 h and infected with I130A-β2-chimerin-AdV (25 m.o.i.) for 16 h. Cells were then harvested using TRIzol reagent for total RNA extraction and Q-PCR analysis as described under "Experimental Procedures." Data are presented as mean ± S.E. (n = 3).

Cells	β2-Chimerin mRNA (copies/10^5 GAPDH copies)
HMT-3522	4527 ± 862
MCF-10A	625 ± 16
MCF-7	3.3 ± 0.2
MCF-7 + I130A-β2-chimerin-AdV	5902 ± 1414

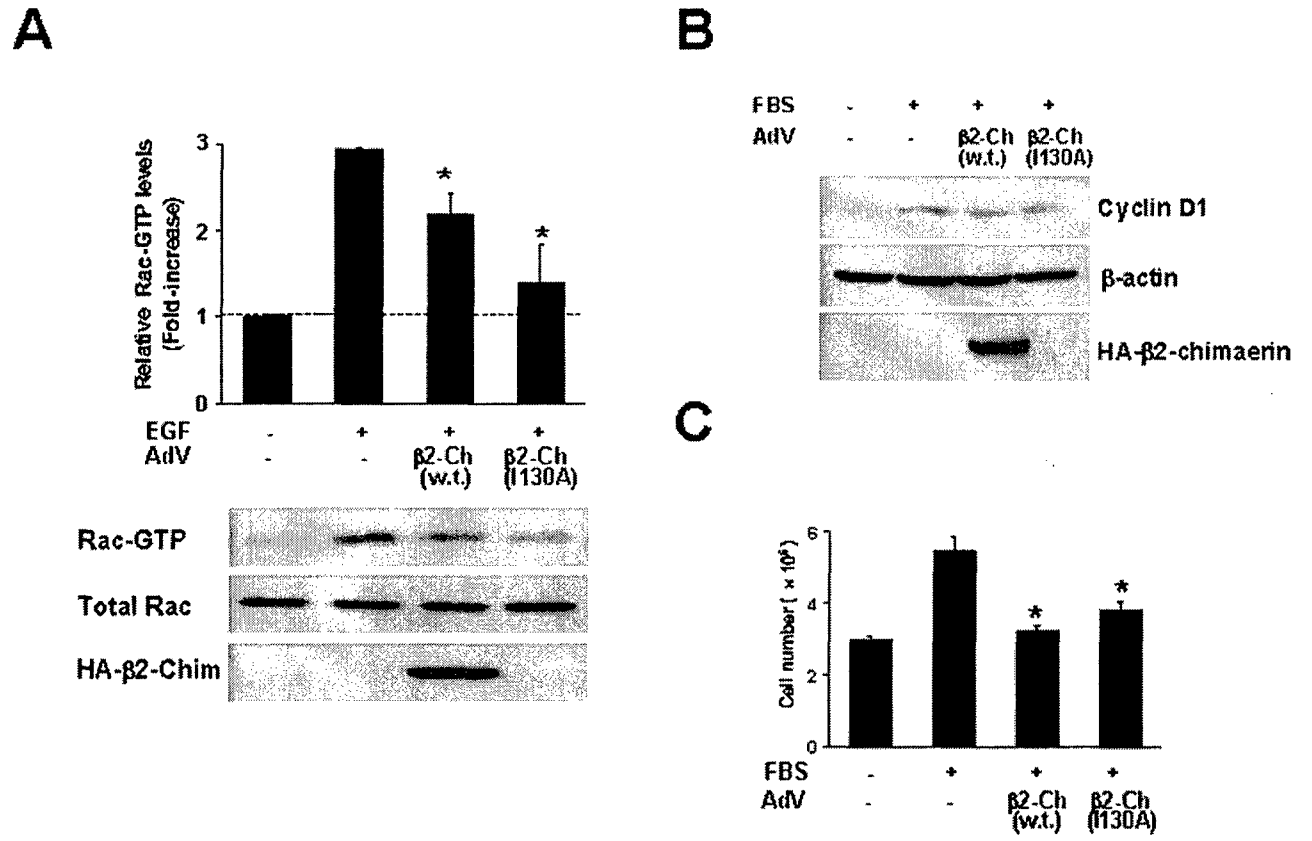

FIG. 8. **The active mutant I130A-β2-chimerin inhibits Rac-GTP levels, cyclin D1 expression, and proliferation.** *Panel A*, after 8 h of serum starvation, cells were infected for 16 h with I130A-β2-chimerin-AdV (*β2-Ch(I130A)*) (25 m.o.i.). Wild-type β2-chimerin-AdV (*β2-Ch-AdV(w.t.)*) (100 m.o.i.) was used as a control. Cells were then stimulated with EGF (100 ng/ml, 1 min). Rac-GTP levels were determined using a PBD pull-down assay, as described under "Experimental Procedures," Densitometric analysis of the inhibitory effect of β2-chimerin on EGF-induced Rac activation was normalized to the corresponding total Rac levels in each case. Data are presented as mean ± S.D. (n = 3). *, $p < 0.05$ compared with cells stimulated with EGF or without β2-chimerin-AdV infection. *Panels B* and *C*, MCF-7 cells were infected as described in *panel A*. After 24 h of serum starvation, cells were stimulated with 10% FBS for 24 h. The expression of cyclin D1 and HA-β2-chimerin was determined by Western blot (*panel B*). Cell proliferation was examined by counting cell number using a hemacytometer (*panel C*). Data are presented as mean ± S.D. (n = 3). *, $p < 0.05$ compared with cells stimulated with 10% FBS without HA-β2-chimerin-AdV infection.

significantly underexpressed in 18% human hepatocellular carcinoma. It is conceivable that the down-regulation of β2-chimerin in breast cancer cells may contribute, at least in part, to the progression of the disease. Early studies in glioma models have identified β2-chimerin as a gene that is significantly down-regulated in high-grade gliomas compared with normal brain and low-grade astrocytomas (8). Down-regulation of β2-chimerin in advanced stages of the disease could contribute to the enhanced proliferation and metastatic dissemination of glioma cells due to dysregulation of Rac activity. Along the same lines, we have recently found using tissue microarrays that β2-chimerin expression is reduced by ~60% in benign duodenal adenomas and ~80% in duodenal adenocarcinomas when compared with normal tissues.[3] β-GAP significantly inhibits cell migration as well as tumor growth, invasiveness, and metastatic dissemination *in vivo* (32), suggesting that specific inhibition of Rac by β2-chimerin may impinge on various steps of malignant transformation. Although more extensive studies would be required to establish whether this Rac-GAP may serve as a prognostic marker, this body of evidence suggests that down-regulation of β2-chimerin expression may contribute to breast cancer progression. This may also be relevant in tissues that express high levels of β2-chimerin, including brain, pancreas, and intestine.

Adenoviral delivery of β2-chimerin, β-GAP, or I130A-β2-chimerin, but not ΔEIE-β2-chimerin, significantly impairs proliferation and elevations in Rac-GTP levels in MCF-7 breast cancer cells, suggesting an essential role for chimerin Rac-GAP activity in these effects. β2-Chimerin also impairs heregulin β1-induced Rac activation and proliferation in breast cancer cells.[4] This highlights the potential relevance of β2-chimerin as a general negative regulator of growth factor-mediated mitogenic responses. Moreover, we have recently observed that β2-chimerin RNAi in HeLa cells leads to a significant potentiation of EGF-induced Rac activation.[2] Our results also emphasize the importance of Rac in cell cycle control, as previously reported using constitutively active and dominant-negative Rac1 mutants (42–44). A dominant-negative N17Rac1 mutant impairs serum-induced DNA synthesis in fibroblasts and has been reported to causes cell growth arrest in G_1 (21) or G_2/M (45). Although the specificity of dominant-negative Rac mutants may be questioned, our experiments revealed that the inhibition of Rac activity with a specific Rac-GAP leads to G_1/S arrest in MCF-7 breast cancer cells, an effect that strongly correlates with the reduction in cyclin D1 and pRb phosphorylation. Rac regulates cyclin D1 expression, probably at multiple levels, depending on the experimental condition and cell type. For example, dominant-negative and constitutively active forms of Rac1 regulate cyclin D1 promoter activity in smooth muscle cells through Rac-dependent generation of reactive oxygen species (24). A role for the NF-κB pathway downstream of Rac has also been described in NIH 3T3 cells (23). Regulation of the cyclin D1 messenger by Rac at a translational level has also been reported (21, 25). Activated Rac is capable of enhancing pRb phosphorylation and E2F-mediated transcription of genes required for S phase entry and DNA replication (46). It has also been proposed that Rac integrates signals from specific integrins and growth factors to promote the synthesis of cyclin D1 and tumor cell survival (24, 25, 47). The insensitivity of V12Rac1-expressing MCF-7 cells to β2-chimerin-induced reduction in cyclin D1 levels and Rb phosphorylation, as well as the rescue of the β2-chimerin effect by ectopic expression of cyclin D1, further supports the Rac-cyclin D1-G_1/S progression link.

An emerging paradigm is that β2-chimerin can be regulated by cell surface receptors, as it is well known for Ras-GAPs (48). Receptors coupled to the generation of the lipid second messenger DAG, such as the EGF receptor, control the activity of β2-chimerin both by positional and allosteric mechanisms,[2] which substantiates the concept of DAG divergence via the activation of "non-PKC" pathways. These DAG-regulated mechanisms, as well as the selectivity of β2-chimerin for the Rac GTPase, have been further validated by the recently solved three-dimensional structure of this Rac-GAP (33). Our hypothesis is that, in the context of receptors such as the EGF or platelet-derived growth factor receptor, β2-chimerins represent a DAG-regulated negative loop that self-limits Rac activation and Rac-mediated responses. Our focus now is to elucidate the molecular basis of such lipid regulation, which will provide further insight into the receptor-mediated control of chimerin function and G_1/S cell cycle progression in breast cancer and other diseases.

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[3] C. Yang and M. G. Kazanietz, unpublished observations.

[4] C. Yang and M. G. Kazanietz, manuscript in preparation.

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Essential role for Rac1 in Heregulin β1-induced Mitogenic Signaling in Human Breast Cancer Cells

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The ErbB family of tyrosine kinase receptors comprises 4 members (EGFR or ErbB1, Her2 or ErbB2, ErbB3 and ErbB4) which play important roles in the progression of various types of cancers, including breast, prostate and colon cancer. Heregulin β1 (HRG) belongs to the family of neuregulins, a group of peptide ligands for the ErbB3 and ErbB4 receptors. How HRG causes the activation of PI3K-Akt and MAPKs to control cell survival and proliferation is not fully understood. We explored whether Rho GTPases play critical roles in HRG-triggered mitogenic signaling. Using a PBD pull down approach, we determined that HRG activates Rac1 in a dose- and time-dependent manner in MCF-7 and T-47D breast cancer cells. HRG-induced Rac1 activation showed a striking different kinetics from EGF-triggered activation of Rac1 in these two cell lines. While EGF-induced Rac1 activation peaked at 1-2 min and returned to basal within 15-30 min, HRG-triggered activation of Rac1 peaked at 5-10 min and still remained high 60 min after stimulation. HRG also caused sustained activation of Cdc42 and RhoA with a similar time-course. By using pharmacological inhibitors, specific blocking antibodies and small interference RNA (siRNA) for individual ErbB receptors, it was determined that the activation of Rac1 by HRG is mediated by ErbB2 and ErbB3 receptors, and that there was an essential requirement for the EGFR in the HRG effect. HRG-induced Rac1 activation was dependent on PI3K but not on Src, as it was impaired by wortmannin but not by PP2. The kinetics of HRG- and EGF-induced Rac1 activation strongly correlated with that for the activation of Erk1/2, JNK and p38 MAPKs. Inactivation of Rac1 by adenoviral delivery of beta2-chimaerin, a Rac-GAP, impaired HRG-induced activation of MAPKs. Moreover, beta2-chimaerin, which did not affect Cdc42 and RhoA activation, also inhibited HRG-induced breast cancer cell proliferation. On the other hand, expression of a constitutively active Rac mutant (V12Rac1) in MCF-7 cells rescued the inhibitory effect of beta2-chimaerin on cell proliferation. These results suggest that Rac1 is an important mediator of HRG mitogenic signaling in breast cancer cells and highlight the complexity in HRG responses via activation of multiple tyrosine-kinase receptors (The U.S. Army Medical Research and Material Command under DAMD 17-03-1-0469 supported this work.).

HEREGULIN β1-INDUCED RAC ACTIVATION PROMOTES BREAST CANCER CELL PROLIFERATION

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Heregulin β1 (HRG) belongs to the family of neuregulins, a group of epidermal growth factor (EGF)-like peptide ligands for the ErbB3 and ErbB4 receptors. It has been found that HRG1 is overexpressed in many kinds of human cancers including breast cancer, and that it promotes breast cancer cell proliferation. The ErbB family of tyrosine kinase receptors comprises 4 members (EGFR or ErbB1, Her2 or ErbB2, ErbB3 and ErbB4) which play important roles in the progression of various types of cancers including breast cancer. How HRG causes the activation of PI3K-Akt and mitogen-activated protein kinases (MAPKs) to control breast cancer cell survival and proliferation is not fully understood. We explored whether Rho GTPases play critical roles in HRG-triggered mitogenic signaling.

Recombinant HRG and two human breast cancer cell lines MCF-7 and T-47D were used for this study. Using a PBD pull down approach, we found that HRG activates Rac1 in a dose- and time-dependent manner in MCF-7 and T-47D breast cancer cells. HRG-induced Rac1 activation showed a striking different kinetics from EGF-triggered activation of Rac1 in these two cell lines. While EGF-induced Rac1 activation peaked at 1-2 min and returned to basal within 15-30 min, HRG-triggered activation of Rac1 peaked at 5-10 min and still remained high 60 min after stimulation. HRG also caused sustained activation of Cdc42 and RhoA with a similar time-course. By using pharmacological inhibitors, specific blocking antibodies and small interference RNA (siRNA) for individual ErbB receptors, it was determined that the activation of Rac1 by HRG is mediated by ErbB2 and ErbB3 receptors, and that there was an essential requirement for the EGFR in the HRG effect. HRG-induced Rac1 activation was dependent on PI3K but not on Src, as it was impaired by wortmannin but not by PP2. The kinetics of HRG- and EGF-induced Rac1 activation strongly correlated with that for the activation of Erk1/2, JNK and p38 MAPKs. Inactivation of Rac1 by adenoviral delivery of beta2-chimaerin, a specific Rac-GAP, impaired HRG-induced activation of MAPKs. Moreover, beta2-chimaerin, which did not affect Cdc42 and RhoA activation, also inhibited HRG-induced breast cancer cell proliferation. On the other hand, expression of a constitutively active Rac mutant (V12Rac1) in MCF-7 cells rescued the inhibitory effect of beta2-chimaerin on cell proliferation.

We conclude that HRG-induced Rac activation is an important mediator of HRG mitogenic signaling in breast cancer cells. These results also indicate that approaches aimed at targeting Rac signaling could open new avenues for breast cancer therapeutics.

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